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(11) **EP 0 791 652 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication: **27.08.1997 Bulletin 1997/35** (51) Int. Cl.⁶: **C12N 15/00, A01K 67/027**

(21) Application number: **97200314.9**

(22) Date of filing: **30.06.1987**

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(30) Priority: **30.06.1986 GB 8615942**

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
87904286.9 / 0 274 489

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Remarks:

This application was filed on 05 - 02 - 1997 as a
divisional application to the application mentioned
under INID code 62.

(54) **Transgenic mammals expressing polypeptide-containing substances in their milk**

(57) Non-human transgenic mammals, adult females of which express proteinaceous substances of interest in their milk, contain in their genetic material a DNA construct comprising DNA encoding the substance of interest and DNA from a milk whey protein gene to direct expression of the substance of interest in the mammary gland. The substance of interest may be harvested from the milk of the adult female mammals. The milk whey protein gene may be β -lactoglobulin.

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Description

This invention relates to a method of producing a substance comprising a polypeptide. More particularly, the invention relates to protein production and to the production of biological materials whose formation is catalysed by enzymic proteins.

Recombinant DNA technology has been used increasingly over the past decade for the production of commercially important biological materials. To this end, the DNA sequences encoding a variety of medically important human proteins have been cloned. These include insulin, plasminogen activator, α_1 -antitrypsin and coagulation factors VIII and IX. At present, even with the emergent recombinant DNA techniques, these proteins are usually purified from blood and tissue, an expensive and time consuming process which may carry the risk of transmitting infectious agents such as those causing AIDS and hepatitis.

Although the expression of DNA sequences in bacteria to produce the desired medically important protein looks an attractive proposition, in practice the bacteria often prove unsatisfactory as hosts because in the bacterial cell foreign proteins are unstable and are not processed correctly.

Recognizing this problem, the expression of cloned genes in mammalian tissue culture has been attempted and has in some instances proved a viable strategy. However batch fermentation of animal cells is an expensive and technically demanding process.

There is therefore a need for a high yield, low cost process for the production of biological substances such as correctly modified eukaryotic polypeptides. The absence of agents that are infectious to humans would be an advantage in such a process.

According to a first aspect of the present invention, there is provided a method of producing a substance comprising a polypeptide, the method comprising incorporating a DNA sequence coding for the polypeptide into a gene of a mammal coding for a milk whey protein in such a way that the DNA sequence is expressed in the mammary gland of the adult female mammal. The substance will generally be recovered from milk of the adult female mammal, either before or preferably after post-translational modification.

As used in this specification, the term "polypeptide" refers to a molecule comprising a chain of amino acids, whether sufficiently long or not to be properly classed as a protein. The polypeptide is preferably sufficiently long to be a protein. The "substance comprising a polypeptide" may be the polypeptide itself, or it may be a modified (for example, glycosylated) polypeptide. Alternatively, or in addition, the substance can be cross linked, by post-translational modification of a polypeptide.

The present invention may be used to produce, for example, peptide hormones, blood coagulation factors (particularly factors VIII and IX) or subunits of them (particularly factors VIII and IX), blood proteins (for example beta-globin) and serum proteins (for example α_1 -antitrypsin) proteins for foodstuffs, including natural or altered milk proteins of the host mammal, or enzymes.

Enzymes produced by the present invention may be able to act on their substrates *in situ* in the mammary gland, and so it can be seen that the present invention encompasses a method of producing a substance which is the reaction product of an enzyme, the method comprising incorporating a DNA sequence coding for the enzyme into a gene of a mammal coding for a milk whey protein in such a way that the DNA sequence is expressed in the mammary gland of the adult female mammal, whereafter it catalyses the formation of the reaction product from one or more substrates of the enzyme. The reaction product will generally be recovered from milk of the adult female mammal.

The DNA sequence coding for the peptide ("the DNA sequence of interest") is preferably incorporated *in vitro* into a milk whey protein gene which is expressible in the mammary gland of an adult female mammal to form a fusion gene and the fusion gene is incorporated into the germline by injection into a fertilized egg of the mammal, whereafter the injected fertilized egg is allowed to develop into an adult female mammal. It is to be expected that not all the injected eggs will develop into adult females expressing the DNA sequence of interest. Apart from anything else approximately half the animals will be male, from which females can be bred in the following generations.

The relevant techniques whereby foreign DNA sequences can be introduced into the mammalian germ line have been developed in the mouse. At present the most efficient route entails the direct microinjection of a few hundred linear molecules of DNA into a pro-nucleus of a fertilized one cell egg, and this is the method of preference in the present invention. Microinjected eggs may then subsequently be transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster *et al* in *Proc Natl Acad Sci* 82 (1985) 4438-4442 that about 25% of the mice that develop inherit one or more copies of the microinjected DNA. From a commercial point of view, it is clearly preferable to use as the host mammal in the present invention a species that is likely to have a larger milk yield. For this reason, domestic livestock animals such as sheep, pigs and cattle are preferred, as will be discussed in more detail below.

Working with large domestic animals requires a considerable investment, particularly in the number of animals required and their cost. While it is normal to obtain up to 30 eggs per superovulated mouse, a ewe gives only three to five. A further problem encountered with farm animals is that the eggs are opaque due to the presence of numerous vesicles, and this makes the identification and successful injection of pronuclei more difficult. Nevertheless, microinjec-

tion of DNA into eggs of rabbits, sheep and pigs has been reported by Hammer *et al*, *Nature* **315** (1985) 680-683, who inserted a metallothionein-growth hormone fusion gene into the germ line of all three mammals.

In the pig, 2,035 eggs were injected and transferred; 192 piglets were born, of which 20 carried the fusion gene. The results were not so encouraging in sheep; 1,032 eggs were injected and transferred, but 73 lambs were born of which only one contained the foreign DNA sequence.

Once integrated into the germ line, the foreign DNA may be expressed in the tissue of choice at high levels to produce a functional protein which can readily be harvested from the animal. To accomplish this, the DNA sequence coding for the peptide of interest will generally be fused to a DNA sequence or sequences that will mediate its expression in a suitable tissue. Although some initial experiments in mice show that tissue specific expression of inserted DNA to be somewhat variable (for example, Lacey *et al*, *Cell* **34** (1983) 343-356), the general consensus now is that correct tissue specific expression of most transfected genes is achievable. See Brinster *et al* *Cell* **27** (1981) 223-231; Swift *et al* *Cell* **38** (1984) 639-646; Shani *Nature* **314** (1985) 283-286; and Magram *et al* *Nature* **315** (1985) 338-340. For correct tissue specificity, it does appear to be important to remove all the prokaryote vector sequences, which are used in the cloning of DNA sequence of interest, prior to microinjection (Krumlauf *et al* *Mol. Cell Biol.* **5** (1985) 1639-1648).

While these publications do indicate that certain different tissues can be targeted in the construction of a transgenic animal, there is no suggestion of the desirability of any particular tissue for commercial production of polypeptide-containing substances. Neither do they suggest any particular gene which is expressed in a given tissue and which would serve to target the expression of the inserted DNA.

A number of factors must be taken into consideration with regard to the substance comprising the peptide and the tissue to which its expression is to be directed. Many proteins require extensive post-translational modification in order to exhibit full biological activity. For example, factor IX requires gamma-carboxylation of a specific subset of glutamic acid residues for biological activity (De Scipio and Davie, *Biochemistry* **18** 899-904 (1979)). Liver, which is the site of natural synthesis of factor IX, is proficient in performing this modification. Fibroblasts are capable of carrying out gamma carboxylation of factor IX, although less efficiently: de la Salle *et al* *Nature* **316** 268-270 (1985). However, certain proteins may be correctly modified only if synthesised in a specific tissue and, in some cases, it may be necessary to tailor the site of expression to the requirements of the proteins produced. It is believed that the use of the mammary gland as a tissue for expression overcomes, either wholly or to a satisfactory degree, this potential source of difficulty.

Harvesting from body fluids as opposed to solid tissue is desirable, because such routes are by and large renewable, and most proteins of biomedical importance are themselves secreted into body fluids. Secretion into the bloodstream is a possible route, either from liver or B lymphocytes, but the coagulant properties of blood and the presence of biologically active peptides and antigenic molecules may prove a hindrance to subsequent downstream processing.

The above difficulties may be overcome in accordance with the present invention by the use of the mammary gland as a tissue of expression. Milk is readily collected, available in large quantities and well characterized biochemically. Further, the major milk proteins are present in milk at high concentrations (from about 1 to 15 g/l).

There are four species of common farm animal in many countries of the world: pigs, goats, sheep and cattle, that is to say members of the family *Suidae*, the genus *Capra*, the genus *Ovis* and the genus *Bos*. Domesticated cattle are generally of the species *Taurus*.

While the present invention is not in its broadest aspect restricted to any particular species, genus or family of mammals, pigs, goats, sheep and cattle are preferred. Each has advantages and disadvantages in the context of the present invention. Some of these considerations stem from the fact that, according to a preferred aspect of the present invention, the DNA sequence coding for the polypeptide is incorporated *in vitro* into a whey protein gene which is expressible in the mammary gland of an adult female mammal to form a fusion gene and the fusion gene is injected into a fertilized egg of the mammal, whereafter the injected fertilized egg is allowed to develop into an adult female mammal.

Although the high milk yield obtained from cattle is a factor tending to suggest that the use of cattle would be preferred, the technical difficulties in manipulating the bovine embryo are greater than in the case of sheep, for example. Furthermore, the sheer cost of experiments involving cattle mean that the other three preferred species of domestic farm animals are the ones of choice.

Table 1 below shows how pigs, sheep and cattle compare with each other for the purposes of use in the present invention.

TABLE 1

Comparison of Different Livestock for Embryo Manipulation and Milk Production.			
	PIG	SHEEP	CATTLE
Possibility of controlling time of follicle maturation	Yes	(Yes)	(No)
No. of oocytes per animal; no superovulation	10	1 to 3	1
No. of oocytes per animal; superovulated	15 to 20	4 to 10	possibly 6
Visualisation of pronuclei	(Yes)	(Yes)	(No)
Seasonal breeding	No	Yes	No
Relative cost per embryo transfer, including nominal cost of animals, per embryo	1.0	1.8	110
Milk yield, litres/day at peak production	n.a. 1	to 3*	5 to 30*

* Depending on breed. n.a. = data not available

The significance of the visualisability of the pronuclei is that it is a preferred feature of the present invention to inject the fusion gene containing the DNA sequence coding for the peptide into the pronucleus of the fertilised egg.

Milk yield data for pigs are not readily available, but in sheep, the rate of milk production falls within the range of from 1 to 3 litres per day, depending on the breed. It is of note that specialized equipment for harvesting milk from sheep is available from commercial suppliers, as of course it is for cattle. Sheep are therefore the animals of choice, in particular dairy sheep such as East Frieslands. A strain obtained by crossing a suitable high milk producing line within Blackface sheep is seen as a viable alternative.

The lactating mammary gland is a highly specialized organ comprising an extensive system of ducts that drain complex lobules of secretory cells. Mammary cells are adapted in many ways to high rates of secretion. For example, they have specialized transport mechanisms that ensure the efficient uptake of precursors from the blood, and an extensive system of intracellular membranes (rough endoplasmic reticulum, Golgi apparatus etc) that enable high rates of protein synthesis, post-translational modification and export from the cell.

The mammary gland is dependent on hormones for all aspects of its growth and function. Review articles on the mammary gland include those by Topper and Freeman, *Physiol Rev* **60** 1049-1106 (1980) and Forsyth in "The Biochemistry of Lactation", Mepham (Ed), Elsevier (1982) 309-349. The hormones affecting the mammary gland mediate the striking changes that occur in the gland during pregnancy and lactation. In the ewe, for example, this leads to a near-doubling in total cell number as well as to changes in the proportions of the various cell types and to the terminal differentiation of the secretory cells.

The mammary gland secretes a number of different proteins into the milk. There are qualitative and quantitative differences in the composition of milk from different species, although a general distinction can be made between the caseins and the soluble (whey) proteins (see Jenness in "Developments in Dairy Chemistry, I", Fox (Ed) Elsevier (1982) 87-109). The major ruminant whey proteins that are synthesised in the mammary gland are alpha-lactalbumin and beta-lactoglobulin.

There are three major types of casein. These are alpha-casein, beta-casein and kappa-casein and appear in most of the species characterized. In milk, they serve to sequester calcium with which they are aggregated in the form of micelles. The function of beta-lactoglobulin, the major whey protein in ruminants, is unknown, although it appears to interact with kappa-casein (Brunner, *J. Dairy Sci.* **64** 1038-1054 (1981)). Alpha-lactalbumin is an essential cofactor in the conversion of glucose and galactose to lactose (Brew, *Nature* **223** 671-672 (1969)). Table 2 shows the protein composition of various milks.

TABLE 2

Protein composition of Various Milks

g/litre	Bovine	Ovine	Murine	Human
CASEINS			7	
Alpha S1	10	12)
Alpha S2	3.4	3.8) 0.4
Beta	10	16		3
Kappa	3.9	4.6		1
MAJOR WHEY PROTEINS				
Alpha lactalbumin	1	0.8	trace	1.6
Beta lactoglobulin	3	2.8	no	no
Whey acidic protein	no	no	2	no
OTHER WHEY PROTEINS				
Serum albumin	0.4	.	.	0.4
Lysozyme	trace	.	.	0.4
Lactoferrin	0.1	.	.	1.4
Immunoglobulins	0.7	.	.	1.4

(Data compiled from various sources)

For the most part it is believed that the different milk proteins are encoded by single copy genes (Mercier and Gaye in "The Biochemistry of Lactation", Mephram (Ed) Elsevier (1983) 177-225). The casein genes appear to be linked both in the mouse (Rosen *et al* Biochem Soc Trans. 9 112 (1982)) and in the cow (Grossclaude Proc. 16th Intl. Conf. Animal Blood Groups Biochem. Polymorphy. 1 54-59 (1979)). Because the casein genes appear to be expressed at a far higher level than the whey protein genes, it would naturally be thought that the casein genes would be the genes of choice in which to incorporate the foreign DNA for expression. However, contrary to the general expectation, the present invention provides that the DNA sequence coding for the peptide of interest be incorporated into a gene coding for a whey protein.

The gene for rat alpha-lactalbumin comprises four exons and encompasses 2.5 kb of chromosomal DNA (Qasba and Safaya Nature 311 377-380 (1984)). The gene for bovine alpha-lactalbumin appears to be similarly organised. It has now been discovered that ovine beta-lactoglobulin is most probably a single copy gene and comprises seven exons within a 4.9 kb transcription unit: this gene is the gene of preference for use in the present invention.

Changes in the levels of milk protein mRNAs during mammary development in the rat (Nakhasi and Qasba, J. Biol. Chem 254 6016-6025 (1979)), rabbit (Shuster *et al* Eur. J. Biochem. 71 193-199 (1976)) and mouse (Pauley *et al* Nature 275 455-457 (1978)) have been assessed using both *in vitro* translation and cDNA hybridisation. The mammary gland accumulates some 80,000 to 100,000 molecules of milk protein-specific mRNAs resulting from a coordinated mechanism comprising both a high rate of milk protein mRNA synthesis and an efficient stabilisation of these molecules (Mercier and Gaye, *op. Cit.* and Guyette *et al* Cell 17 1013-1073 (1979)). There are, however, exceptions. In the ewe beta-casein makes up 45% of the total milk protein, yet only 17% of cDNA clones isolated from a cDNA library corresponded to this gene (Mercier *et al* Biochimie 67 959-971 (1985)). In the rabbit, the amount of alpha-lactalbumin sequestered into microsomal vesicles was much lower than that expected from the rate of protein synthesis in the absence of microsomal vesicles, possibly suggesting that the signal peptide does not have an optimal configuration (Gaye *et al* Biochimie 64 173-184 (1982)). The primary translation products of many of the milk protein genes are exten-

sively modified. For example, alpha-lactalbumin is N-glycosylated, kappa-casein is O-glycosylated and alpha- and beta-casein are O-phosphorylated. In addition to its role in post-translational modification, the Golgi complex is also involved in condensing and packaging caseins into micelles. It is a possibility that the caseins and the whey proteins are exported from the cell by different routes. In this regard it is of interest to note that, in both inter- and intra-specific comparisons, the casein signal peptide (in comparison to a large proportion of the rest of the molecules) is highly conserved, suggesting that it may play a role in targeting the nascent peptide into the correct secretory pathway (Mephram *et al op cit*). As described above, milk protein genes are abundantly expressed in the lactating mammary gland. In the ewe, for example, alpha_{S1}-casein mRNA accounts for about 30%, and beta-lactoglobulin accounts for about 5%, of polyA+RNA (Mercier *et al* (1985) *op cit*). Given that these transcripts originate from single copy genes, then these levels indicate high rates of transcription and efficient mRNA stabilisation (Teyssot and Houdebine, *Eur. J. Biochem.* **110** 263-272 (1980)). Specific mRNA stabilisation may be mediated by sequences present in the mature mRNA. In preferred embodiments of the present invention, similar levels of expression of the DNA sequence that is inserted into the ovine germ line may be achievable. This is accomplished by linking it to the DNA sequences associated with a milk whey protein gene that mediate the high levels of tissue-specific expression and mRNA stabilisation: beta-lactoglobulin is the gene of preference.

Transferring new genes into mice is now a routine procedure. In transgenic mice that exhibit high levels of tissue specific expression of a foreign gene, the exogenous DNA comprises not only the structural gene but also the 5' and 3' flanking sequences. Although there is an abundance of evidence suggesting that many important regulatory elements are located 5' to the mRNA cap site (see for example McKnight and Kingsbury, *Science* **217** 316-324 (1982); Payvar *et al Cell* **35** 381-392 (1983); Renkawitz *et al Cell* **37** 503-510 (1984); Karin *et al Nature* **308** 513-518 (1984)) it is also evident that important regulatory sequences, particularly those mediating tissue-specific expression, may reside within the structural gene or even 3' to it (Charnay *et al Cell* **38** 251-263 (1984); Gillies *et al Cell* **33** 717-728 (1983)). Furthermore exonic sequences (i.e. those present in mature mRNA) may contain sequences that mediate mRNA stability. Table 3 illustrates some details of tissue-specific expression of foreign genes in mice.

TABLE 3

Authors	Construct	No. egg re- implanted	DNA copies injected	Transgenic Progeny	Gemline Transmission	Expression	Site of Expression
Gordon et al <u>PNAS</u> 77 7380-7384 (1980)	PR-SV40 + havTK Cir, Vec	'sev 100s'	500- 12,000	2/173	nd	nd	
E. Wagner et al <u>PNAS</u> 78 5016-5020 (1981)	hBG, hav TK	ns	2,500	5/33	nd	+	
Brinster et al <u>Cell</u> 27 223-231 (1981)	PR-mYT + havTK Cir, Vec, U1700	240	200	7/41	nd	4/7	liver, kidney
T. Wagner et al <u>PNAS</u> 78 6376-6380 (1981)	raBG 1. Cir, Vec 2. Lin, Nov	211	20,000	7/46	+	+	
Costantini & Lacy Nature 294 92-94 (1981)	raBG Lin, Vec, U14000+	ns	100- 1000	9/24	4/4	nd	
Gordon & Ruddle <u>Science</u> 214 1244- 1246 (1981)	hIFN (alpha) (cDNA) Cir, Vec	ns	10,000	1/10	+	nd	

Table 3 cont'd

Palmiter et al Cell 29 701-71- (1982)	PR-mMT + hsvTK Cir/Lin, Vec/Nov (U1700)	ns	200- 600	10/69	+	7/10	primarily liver
Palmiter et al Nature 300 611-615 (1982)	PR-mMT + rGH	170	600	7/21	nd	+	primarily liver, express hypervariable
McKnight et al Cell 34 335-341 (1983)	cTransferrin 1. Cir, Vec 2. Lin, Vec U2000	ns	1. 500 2. 140	3/19	+	6/7	liver, kidney (tail, testes weak expression)
Lacy et al Cell 34 343-356 (1983)	raBG Lin, Vec, U14000+	ns	100- 1,000	9/24	8/9	2/9	muscle or testis
Palmiter et al Science 222 809-814 (1983)	PR-mT + hGH Lin (Vec) U230	1000	1,000	33/101	nd	+	primarily liver
Brinster et al Nature 306 332- 336 (1983), Storb et al Nature 310 238- 241	mIgK (lit)	192	400	6/11	4/4	+	spleen/B- lymphocytes (1 x kidney)
Hammet et al Nature 311 65-67 (1984)	PR-mMT + rGH or hGH Lin, Nov U185	494	300- 600	32/ns 7/41	7/7	+	nd

Table 3 cont'd

Brinster et al Cell 37 367-379 (1984)	PR-SV40 + svT Lin, Vec	925	240- 830	25/95	-	+	brain tumours
Swift et al Cell 38 639-646 (1984)	rElastase type I Lin, (Vec) U7000	320	200	7/37	7/7	5/5	exclusively pancreas
Grosschedl et al Cell 38 647-658 (1984)	mIgM hev (functionally re- arranged) Lin, Vec, U200	284	50	5/13-23	3/4	4/5	primarily (exclusively) B and T lymphoc
Stewart et al Cell 38 627-637 (1984)	PR-mMTV + mMYC Lin, Vec, U2000	ns	500	13	nd	11/12	primarily salivary gland (mammary tumour)
Ornitz et al Nature 313 600-602 (1985)	PR-rElastase type I + hGH Lin, Vec, U200	ns	nd	22/131	nd	13/18	exclusively pancreas
Shani Nature 314 283-286 (1985)	rHyosin lit-2; Lin, Vec, U1200	ns	500	4/26	3/4 (1 mosaic)	2/4	exclusively skeletal mu
Rusconi & Kohler Nature 314 330- 334 (1985)	rIgM hev & lit (K) (functionally rearranged) Cir, Vec, U3000- U5000	ns	20- 100	5/13	4/5 (1 mosaic)	5/5	B lymphocytes
Chada et al Nature 314 377- 380 (1985)	PR-mBG + hBG Cir, Vec or Lin, Nov U1200	ns	400- 800	10	nd	4/10	primarily erythroid cells low expression

Table 3 cont'd

Hanahan Nature 315 115-121 (1985)	PR-rinsulin II + svT Lin, Vec (Lin, Nov)	ns	20- 150	5	5/5	+	exclusively pancreas (B cell tumours)
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Cir = injected with circular DNA; Lin = DNA linearised before transfer; Nov = vector removed before transfer; Vec = Vector present during transfer; U = upstream sequences; U1000 = 1000 bp of U sequences

c = chicken; h = human; hsv = herpes simplex virus; m = mouse; r = rat; ra = rabbit;

sv = simian virus 40

BG = bea globin; GH = growth hormone; IFN = interferon; Ig = immunoglobulin; PR = promoter;

T = T antigen;

TK = thymidine kinase; hev = heavy chain; lit = light chain; nd = not determined; us = not specified.

It is for the above reason that the above preferred fusion genes that will be used to direct the expression of the DNA sequence coding for the peptide of interest will comprise one or more of: a promoter; a start site for transcription; one or more (presumed) distal 5' regulatory sequences of a milk protein gene; structural milk protein gene sequences; 3'

sequences flanking a milk protein gene. The most preferred fusion genes comprise sequences of all these types.

A fusion gene of choice will consist of a cDNA sequence coding for the peptide of interest inserted into the first exon of a whey protein gene. It is preferred that several kb of the 5' flanking sequences of the milk protein gene will be included in such a fusion gene. In these constructs, secretion of the peptide of interest will preferably be mediated by its own signal peptide; it is therefore preferred that the fusion gene contain a signal peptide for the peptide of interest. However, a tissue-specific signal peptide may be important for targeting the nascent peptide into the correct secretory pathway (as discussed above). Therefore, it is particularly preferred that DNA sequences encoding the signal peptide of the whey protein gene will be precisely fused to the DNA sequences of the insert that encode the N-terminal amino acid of the mature protein. The 3' end of the insert will preferably terminate after its stop codon, but before its own message cleavage and polyadenylation site. Downstream of the site of insertion, the rest of the structural gene will generally be retained, as well as some 3' flanking sequences.

In a transgenic animal, such a construct should maximise the chances of obtaining high levels of mammary gland-specific expression. The primary transcript should be correctly polyadenylated and spliced at the sites retained in the whey protein gene. The mature message should contain sequences for efficient stabilisation of mRNA. In constructs employing the milk gene signal peptide, the mature mRNA should be translated to yield a fusion pre-peptide in which the signal peptide derived from the milk protein gene efficiently directs the secretion of the mature peptide encoded by the cDNA insert.

Ovine beta-lactoglobulin is, as discussed above the whey protein gene of choice with which to "entrain" cDNA coding for the peptide of interest. In sheep, beta-lactoglobulin is the most abundantly expressed whey protein in the mammary gland and its mRNA comprises about 8% of the total polyA+ RNA. The gene has been well characterized and used to elaborate fusion constructs with cDNA sequences encoding human factor IX and human alpha1 antitrypsin.

According to a second aspect of the present invention, there is provided a genetic construct comprising a DNA sequence encoding a peptide incorporated into a gene of a mammal coding for a milk whey protein in such a way that the DNA sequence is expressible in the mammary gland of the adult female mammal.

According to a third aspect of the present invention, there is provided an animal cell including a genetic construct as described above. The animal cell may be an embryo cell.

According to a fourth aspect of the present invention, there is provided a plasmid comprising a genetic construct as described above.

Preferred features of the second, third and fourth aspects are as described above for the first aspect, mutatis mutandis.

For the better understanding of the present invention, a number of examples will now be given. In the examples, reference will be made to the drawings in which:

FIGURES 1 to 4 show schematically the basic steps in the elaboration of a beta-lactoglobulin fusion gene in a method in accordance with the present invention;

FIGURE 5 shows a Southern blot analysis of sheep DNA;

FIGURE 5a shows a Southern blot analysis of sheep DNA and illustrates the inheritance of the beta-lactoglobulin fusion gene by the real generation.

FIGURE 6 shows an SDS-PAGE analysis of murine and ovine whey proteins; and

FIGURE 7 shows a Western blot analysis of sheep and mouse proteins.

FIGURE 8 shows a RIA of milk obtained from transgenic ewe-sheep carrying the beta-lactoglobulin fusion gene as described.

EXAMPLE 1

A. RECOMBINANT DNA PROCEDURES

Where not specifically detailed, recombinant DNA procedures were after Maniatis *et al* ("Molecular Cloning" Cold Spring Harbor (1982)) and "Methods in Enzymology" Vols 68, 100 and 101 (Wu, Grossman and Moldave, Eds.), Academic Press; and, unless specifically stated, all chemicals were purchased from BDH Chemicals Ltd, Poole, Dorset, England or the Sigma Chemical Company, Poole, Dorset, England.

CONSTRUCTION OF OVINE BETA-LACTOGLOBULIN FUSION GENESPreparation of Sheep Spleen DNA

5 Spleen tissue was procured from a freshly slaughtered Blackface/Suffolk lamb and nuclei were isolated essentially as described by Burch and Weintraub *Cell* **33** 65 (1983). Nuclear pellets were lysed in 0.3M NaCl, 10mM Tris.HCl, 10mM EDTA, 1% SDS pH 7.4 and 400 mcg/ml Proteinase K (Sigma Co, Fancy Road, Poole, Dorset BH17 7NH) and incubated for two hours at 37°C. Repeated phenol/chloroform extractions were performed until the preparation was completely deproteinised. The DNA was ethanol precipitated and spooled out using a glass rod, washed with 70% EtOH/30% TE (TE = 10mM Tris.HCl, 1mM EDTA pH 8.0), dried in air and resuspended in TE to a concentration of 1 mg/ml.

Construction of Sheep Spleen DNA Lambda Fusion Genes

15 The lambda phage EMBL3 (Frischauf *et al* *J. Mol. Biol.* **170** 827 (1983)) was employed to construct the genomic library. 30 mcg of bacteriophage DNA were digested with 5-fold excesses of the restriction enzymes *Eco*RI and *Bam*HI (supplied by Amersham International plc, Lincoln Place, Green End, Aylesbury, Buckinghamshire, England) using the conditions recommended by the manufacturer. After digestion, spermine hydrochloride was added to a concentration of 5mM to precipitate the lambda DNA. After incubation for one hour on ice the DNA was pelleted at 10,000g for 15 minutes in a bench microfuge, washed in 70% EtOH, 300mM NaAc, 100mM MgCl₂, repelleted and finally resuspended in TE at a concentration of 1 mg/ml.

Sheep DNA was partially digested with the restriction enzyme *Sau*3A (Amersham). 100 mcg aliquots of the sheep DNA were digested with varying amounts of *Sau*3A [from 5-40 units] for 20 minutes at 37°C. The reactions were stopped by the addition of EDTA to 15mM. The degree of digestion was assessed by electrophoresis on 0.6% agarose gels. Suitably digested samples were pooled and loaded onto 38.0ml 10-40% sucrose gradients made up in 1M NaCl, 20mM Tris.HCl, 5mM EDTA at pH 8.0. These gradients were centrifuged in a Beckmann SW 28 rotor at 26,000 rpm for 24 hours. The sucrose gradients were fractionated from the top and 1ml fractions collected. The size distribution of DNA molecules in each fraction was assessed by agarose gel electrophoresis, and fractions containing DNA molecules from 14-21 kb in size pooled. After a two-fold dilution in TE 2 volumes of EtOH were added and the DNA precipitated overnight at -20°C. The DNA was subsequently resuspended in TE to a concentration of 300 mcg/ml.

7.5 mcg of *Bam*HI/*Eco*RI cut EMBL3 and 2.5 mcg of sheep spleen DNA which had been partially digested with *Sau*3A were mixed together in 50 mcl of a solution containing 60mM Tris.HCl, 6mM MgCl₂, 10mM DTT, 0.01% gelatin, 0.25mM rATP and 25 units of T₄ DNA ligase (Boehringer Company, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex) and incubated overnight at 14°C.

35 After ligation 1 mcg aliquots of the DNA were packaged *in vitro* using a kit purchased from Amersham following the recommended procedure of the manufacturer. The packaged library was titred on *E. coli* strain ED 8654. The estimated size of the library was 5.7 X 10⁵ plaque forming units (pfu's). Immediately after titration, aliquots of the unamplified library were plated onto 10 x 22cm² petri dishes (megaplates) using *E. coli* strain ED 8654 at a density of approximately 50,000 pfu's/plate.

Screening the Lambda Genomic Library

Plaque-lifts from the mega-plates were performed according to the method of Benton and Davis (*Science* **196** 180 (1977)) onto 20cm² nitrocellulose membranes (Schleicher and Schull, Postfach 4, D-3354, West Germany). A beta-lactoglobulin cDNA clone (p931 - gift of J.C. Mercier, INRA, Jouey-en-Josas, Paris) was nick translated with ³²P dCTP to a specific activity > 10⁸ dpm/mcg, by the method described by Rigby *et al* (*J. Mol. Biol.* **113** 237 (1977)). Beta-lactoglobulin cDNA may be cloned as described by Mercier *et al* in *Biochimie* **67** 959-971 (1985). The sequence of the p931 clone is given by Gaye *et al* in *Biochimie* **68**, 1097-1107 (1986).

Filters were prehybridised, hybridised and washed according to the method of Maniatis *et al* in *Cell* **15** 687 (1978). The final wash was in 1 x SET at 68°C (SET is 0.15M NaCl, 2mM EDTA, 0.03m Tris.HCl pH 8.0). Filters were blotted dry and spotted with ³²P to orientate them before exposure to X-ray film. Regions containing positively hybridising plaques were positioned on the megaplates by reference to the ³²P spots picked using the sterile blunt end of a Pasteur pipette. The initial plaque lifts were titred on *E. coli* ED 8654 and plated onto 15cm diameter Petri dishes at a plaque density of approximately 500/plate. These plates were rescreened by the procedures described above and individual positively hybridising plaques were picked using a toothpick into 1.0ml of phage-buffer (phage buffer is 10mM Tris.HCl, 10mM MgCl₂, 0.01% gelatin, pH 7.4).

Preparation of Cloned Beta-Lactoglobulin DNA

0.4ml of the resuspended phage solution was added to *E. coli* ED 8654 (Borg *et al* *Mol. Gen. Genetics* **146** 199-207 (1976)) and plated out on 9cm diameter Petri dishes to obtain confluent lysis of the bacterial lawn. Confluent plates were obtained from which the top plating agar was scraped off into 10ml of phage buffer and incubated overnight with a few drops of chloroform. The bacterial debris was pelleted by centrifugation at 5000 rpm for five minutes and the phage stocks stored at 4°C. The stocks were titrated on *E. coli* ED 8654 to determine the pfu/ml.

8 x 10⁷ pfu's were absorbed onto 7 x 10⁹ *E. coli* cells in 10ml of 10mM MgSO₄ at 37°C. After 15 minutes, 2.5ml aliquots were added to 100ml L Broth/10mM MgSO₄ in a one litre flask. The bacterial suspension was shaken vigorously for several hours and the OD₅₄₀ was monitored every hour. Lysis, as determined by a fall in the OD₅₄₀, occurred after several hours. When complete, 0.2ml chloroform was added to each 100ml culture and the culture left at 4°C overnight.

The bacterial debris was removed by centrifugation at 10,000 rpm for 15 minutes. 10 mcg/ml RNAase A and 10 mcg/ml DNAase I were added to the supernatant which was then incubated at 37°C for one hour. After this incubation NaCl was added to 40g/litre and polyethylene glycol (PEG) to 10%. The preparation was cooled to 4°C and left for at least two hours to precipitate the phage. The phage pellet was pelleted at 10,000 rpm for 15 minutes and resuspended in 16.0ml of phage buffer. 8.0ml of this suspension was layered upon a step gradient comprising 1.5ml 56% CsCl, 1.5ml 45% CsCl and 2.5ml 31% CsCl (dissolved in phage buffer) in a 14.0ml ultracentrifuge tube. The step gradients were centrifuged at 35,000 rpm for 1.5 hours in a swing-out rotor at 20°C. The phage band was removed with a needle and syringe and, to complete the purification of the phage particles, a second step gradient centrifugation was performed.

The purified phage particles were dialysed into 0.1M NaCl, 10mM Tris.HCl, 1mM EDTA pH 8.0 and then deproteinised by successive extractions with phenol and chloroform. NaCl was added to a final concentration of 0.3M and then the phage DNA precipitated by the addition of 2 volumes of EtOH. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes, washed with 70% EtOH, 30% TE, dried and then resuspended in TE to a final concentration of 200-400 mcg/ml.

Characterisation of Recombinant Beta-Lactoglobulin Clones

0.5 mcg aliquots of the DNA preparations described above were restricted with a variety of restriction enzymes and the products of the single and double digests analysed by electrophoresis on 0.6% and 1% agarose gels. DNA on these gels was transferred to nitrocellulose filters on to Hybond membranes (Amersham International, Little Chalfont, Bucks) by the method of Southern (*J. Mol. Biol.* **98** 503 (1975)) and hybridised to ³²P labelled p931. The procedure used was essentially as described above and the hybridised filters were analysed by autoradiography. Using a variety of restriction enzymes and specific probes from the 5' and 3' ends of p931 a restriction map was constructed in which the size and orientation of the beta-lactoglobulin gene(s) was determined, (see Figure 1).

The identity of the beta-lactoglobulin clones and the precise position of the 5' and 3' ends of the gene were directly confirmed by DNA sequencing. Using suitable restriction sites, fragments were subcloned into plasmid vectors and into M13 vectors. Sequencing was carried out using the dideoxy method of Sanger *et al.* (*PNAS* **74** 5463 (1977)).

Elaboration of Beta-Lactoglobulin Fusion Genes

The strategy used for elaborating fusion genes comprising beta-lactoglobulin and genes of interest to be expressed in the mammary gland is outlined in Figures 1 to 4. The approach utilises sequences derived from a lambda clone, whose isolation and characterisation is described above. The strategy involves insertion of the DNA sequence of interest into the region of DNA corresponding to the 5' untranslated region of beta-lactoglobulin mRNA. Proteins translated from mRNA transcripts of this gene will contain the secretory peptide of the target protein.

The subclone pSS-1tg SE was constructed as shown in Figure 1 by ligating the 1.4 kb *Sph*I-*Eco*RI fragment of lambda phage SS-1 into the vector plasmid pPoly which had also been cut with *Sph*I + *Eco*RI and *E. coli* strain DH1 transformed having been made competent by the procedure of Hanahan and Meselson (*Gene* **10** 63 (1980)). Ampicillin resistant clones were isolated and DNA prepared from them by the method of Birnboim and Doty (*Nuc. Acid Res.* **7** 1513 (1979)).

In Figure 1, the top arrow defines the orientation and extent (approximately 4.9 kb) of the beta-lactoglobulin transcription unit present within lambda SS1; the scale is the same throughout. More generally, in Figures 1 to 4, it should be noted that only relevant restriction sites are shown. Large open boxes represent lambda EMBL3 arms; narrow open boxes represent pPoly; the narrow shaded box represents the target sequence to be expressed; lines represent cloned sheep sequences corresponding to the beta-lactoglobulin gene and its flanking sequences.

pSS-1tgSE was linearised by digestion with the restriction endonuclease *Pvu*II (Figure 2) which cuts at a unique site in the plasmid in a region of DNA corresponding to the 5' untranslated mRNA sequences of beta-lactoglobulin. 5 mcg of completely digested plasmid was dissolved in 0.5M Tris.HCl, 10mM MgCl₂, 1mM MnCl₂, 10mM spermidine, pH

9.0, and treated with 0.01-0.04 units of calf intestinal phosphatase (Boehringer) for 30 minutes at 56°C. The calf intestinal phosphatase was inactivated in 0.5% SDS and the DNA recovered by phenol/chloroform extractions and EtOH precipitation.

A Factor IX DNA clone p5'G3'CVI was procured from Dr. G. Brownlee, Sir William Dunn School of Pathology, University of Oxford, Oxford. This clone contains a 1579 bp insert in a plasmid derived from pAT153 (Twig *et al* *Nature* **283**, 216-218 (1980)). It runs from the *TagI* site at -7 from the presumed mRNA start site to +1572 and contains the entire coding sequence for human Factor IX (Anson *et al* *Embo J.* **3**, 1053-1060 (1984)).

A *NheI*-*HindIII* fragment, comprising 1553 bp of Factor IX sequences was excised and purified from the vector sequences by the methods described below. The *HindIII* and *NheI* ends of this were blunted using Klenow polymerase by the technique described by Maniatis *et al* ("Molecular Cloning" Cold Spring Harbor (1982)) and the fragment ligated into *PvuII* restricted, phosphatased pSS1tgSE (described above) to form pSS1tgSE-Factor IX after transforming *E. coli* DH-1 to ampicillin resistance.

The Factor IX cDNA sequence is hereafter designated TARG, as can be seen in Figure 2.

Plasmid DNA was prepared as described above and checked by digestion with suitable restriction enzymes. The plasmid DNA was digested with *SphI* + *EcoRI*, electrophoresed on a 1% agarose gel containing 0.5 mcg/ml ethidium bromide (Sigma). The relevant *SphI*-*EcoRI* fragment was located by illumination with a UV lamp (Ultra-Violet Products, Inc., San Gabriel, California, USA). A piece of dialysis membrane was inserted in front of the band and the DNA subsequently electrophoresed onto the membrane. The DNA was eluted from the dialysis membrane and isolated by use of an "Elu-tip" [Schleicher and Schull, Postfach 4, D-3354, Dassel, W. Germany], employing the procedure recommended by the manufacturer.

The plasmid pSS1tgXS (Figure 3) was constructed by ligating the *XbaI*-*SalI* fragment of lambda SS-1 into *XbaI*-*SalI* digested pPoly. Clones were isolated and plasmid DNA prepared as previously described. Two DNA fragments were independently isolated from this plasmid or smaller subclones derived from it: a 10.5 kb *SphI* (partial) *HindIII* fragment and a 1.2 kb *EcoRI*-*HindIII* fragment (Figure 4). These fragments were isolated by gel electrophoresis, as described above.

The *SphI*-*EcoRI*, *SphI*-*HindIII*, and the *EcoRI*-*HindIII* fragments were ligated together in approximately equal ratios and the DNA used to transform DH-1. Plasmid DNA (pSS1tgXS-TARG) was prepared as described above and digested with *XbaI* and *SalI* to excise the beta-lactoglobulin fusion gene from the vector. This fragment was purified by gel electrophoresis followed by the use of an "Elu-tip". The ethanol precipitated DNA was pelleted, resuspended in TE and phenol/chloroform extracted and reprecipitated. The DNA was finally resuspended in TE and was employed directly for microinjection.

B. CONSTRUCTION OF TRANSGENIC ANIMALS

Collection of Fertilised Eggs

Procedures were analogous to those described for mice in Gordon and Ruddle in "Methods in Enzymology" (1983) Vol 101 (W. Grossman and Moldave, Eds.) Academic Press pp411 to pp432.

Embryos are recovered from ewes that have been induced to superovulate at an oestrus that is controlled by treatment with progestagen. Mature ewes of proven fertility are treated for 12-16 days with intravaginal sponges that are impregnated with 60mg medroxyprogesterone acetate (Veromix, Upjohn Ltd. Crawley). Equine follicle stimulating hormone [3.5-4.3mg in aqueous solution/ewe] is given in 2 equal intramuscular injections 28 hrs before the end of progestagen treatment and at the time of sponge withdrawal. Ewes are allowed to mate several times at the oestrus that occurs 20-72 hrs after sponge removal. The ewes are observed for the onset of heat at 08.00, 12.00, 16.00 and 20.00 hr daily. Embryos at the 1 to 4 cell stages of development are recovered during surgery 36-72 hrs after the onset of oestrus.

Anaesthesia is induced by intravenous injection of thiopentone sodium [Intraval, May and Baker] and maintained by mixtures of oxygen and nitrous oxide in a semi-closed circuit system. Embryo recovery was carried out by the procedures of Hunter *et al* (*J. Agric. Sci.* **46** 143-149 (1955)). The reproductive tract is exposed through a mid-central incision and a nylon catheter inserted into the oviduct through the fimbria. Medium is introduced into the uterine lumen through a blunted 18-gauge needle and forced through the uterotubal junction and along the oviduct. The embryos are recovered in phosphate-buffered saline containing energy sources and protein [Ovum Culture Medium, Flow Labs, Irvine, Scotland]. During storage and microinjection of eggs this medium is supplemented with 20% foetal calf serum.

Injection of DNA

DNA (1 to 2 mcg/ml) is injected into one pronucleus of single cell eggs or into one or more nuclei of 2 and 4 cell eggs. The eggs are manipulated in a chamber filled with ovum culture medium. The chamber consists of a siliconised microscope slide with glass supports (25mm x 2mm x 3mm) parallel to the long side of the slide. A coverslip is mounted

on top of the supports, the junctions being sealed with silicone grease. The open ends of the chamber are filled with Dow-Corning 200 fluid (50cs) (BDH Chemicals).

Eggs to be injected are held by suction on a blunt glass pipette. Pronuclei or nuclei are visualised using a Nikon Diaphet inverted microscope (Nikon (UK) Ltd, Haybrooke, Halesfield 9, Telford, Shropshire). DNA is injected into pronuclei or nuclei using a micropipette drawn from capillary tubing (borosilicate glass - 1mm external diameter, thin wall with filament - Clark Electromedical Instruments, PO Box 8, Pangbourne, Reading, RG8 7HU) on a microelectrode puller (Campden Instruments, 186 Campden Hill Road, London). The positions of the two microinstruments are controlled using micro-manipulators (Leitz Mechanical Micromanipulators, E. Leitz (Instruments) Ltd, 48 Park Street, Luton, England). The micropipette containing the DNA to be injected is connected via air-tight tubing to a 100 ml glass syringe. Injection is performed by application of pressure using the syringe. Successful injection is indicated by visible swelling of the pronuclei or nuclei. The injected eggs are incubated at room temperature for a minimum of 30 minutes to allow visible degeneration of damaged eggs.

Embryos judged to have survived injection are transferred to un-mated recipient ewes whose oestrus cycles are synchronised with those of egg donors by treatment with progestagen [Veromix, Upjohn Ltd]. Embryos are transferred to the oviduct using fine-drawn mouth-pipettes. Up to 4 embryos are transferred to each ewe. The embryos are distributed between the oviducts.

The body-wall of the ewes is closed with soluble filament [Descon, Davis and Greck] and the skin with Michel clips. Each ewe is given antibiotics [Duphaphen L.A., Duphar, Amersham] at the time of surgery. The Michel clips are removed 10-20 days after surgery.

Development and Growth

After recovery from anaesthesia the ewes are returned to the paddock where they remain throughout pregnancy. Supplementary hay, turnips and concentrates are provided as required. During the third month of pregnancy the number of fetuses is determined by real ultra-sonic scanning. (White *et al* Vet. Rec. **115**, 140-143 (1986)). Management of pregnant ewes is then adjusted to take account of variation in foetal number. As the expected date of parturition approaches the ewes are housed to facilitate supervision and assistance during lambing.

Analysis of Transgenic Lambs

At least 2 weeks after birth 10 ml samples of blood are removed by venous puncture with a hypodermic syringe and collected in a heparinised tube. DNA was prepared from blood samples as follows:- 30 ml of lysis solution (155mM NH₄Cl, 10mM KHCO₃, 1mM EDTA) was added to a 10 ml blood sample and the mixture incubated for 15 minutes on ice. The white blood cells were spun down at 1500g for 10 minutes at 40°C, resuspended in 10ml SE (75mM NaCl, 2mM EDTA) and then washed once in SE. Proteinase K was added to 100 mcg/ml followed by 1ml 20% SDS and the preparation incubated for 4 hours. Repeated phenol/chloroform extractions were performed until the preparation was completely deproteinised. 1/30th vol of 0.3M NaAc-1 vol isopropanol were added to the aqueous phase to precipitate the DNA, which was hooked out, rinsed in 70% EtOH and resuspended in TE.

10mcg aliquots of the DNA preparations were digested with suitable restriction enzymes (eg *EcoRI*) and electrophoresed on 0.8% gels. These gels were analysed by Southern Blotting and hybridisation was performed essentially as described.

Positively hybridising animals, ie those containing the fusion gene (presumed to be integrated at a chromosomal location), are allowed to grow to maturity. Females are mated and, once lactating, their milk analysed for the substance of interest (see Example 7). Positive males are mated and their daughters screened for the exogenous DNA sequences and their milk is subsequently analysed for the substance of interest.

EXAMPLE 2

The procedure of Example 1 is repeated, except that the DNA sequence encoding the polypeptide of interest (the TARG sequence) encodes alpha₁-antitrypsin was obtained from R. Cortese, EMBL, Meyerhofstrasse 1, D-6900 Heidelberg, West Germany. A *TagI-BstNI* fragment comprising 1294 bp of the insert (see Ciliberto *et al* Cell **41** 531-540 (1985)) was excised, purified from this clone and cloned into the *PvuII* site of pSSItgSE by the methods described in Example 1.

EXAMPLE 3

The procedure of Example 1 is repeated, except that instead of the plasmid pPoly, the plasmid pUC18 (Pharmacia Ltd, Pharmacia House, Midsummer Boulevard, Milton Keynes", England) is used instead.

EXAMPLE 4

The procedure of Example 1 is repeated, except that instead of the plasmid pPoly, the plasmid pUC19 (Pharmacia Ltd, Pharmacia House, Midsummer Boulevard, Milton Keynes", England) is used instead.

EXAMPLE 5**Generation of Transgenic Sheep**

The Sall-XbaI fragment, excised from the plasmid pSS1tgXS-FIX (also designated pSS1tgXS-TARG, see Example 1), was injected into sheep eggs. Approximately 200 copies/fertilised egg were injected. From 252 one cell eggs injected and reimplanted into recipient ewes, 52 live lambs were born. Based on the analysis of DNA prepared from blood samples four of these animal were shown to carry the exogenous betalactoglobulin- Factor IX sequences (Table 4).

TABLE 4

Summary of Transgenic Sheep.			
LAMB No.	SEX	CONSTRUCT	COPY No. (Approx)
6LL225	M	BLG-FIX	40
6LL231	F	BLG-FIX	10
6LL239	M	BLG-FIX	1
6LL240	F	BLG-FIX	10
All of the eggs were injected at the pronucleus stage and none were centrifuged.			
Copy numbers were determined by quantitative scanning densitometry.			

Figure 5 is a Southern blot analysis of the sheep DNA. Sheep DNA was prepared as described, digested with the restriction enzymes indicated, electrophoresed on a 0.8% agarose gel and transferred to Hybond membrane (Amersham International, Little Chalfont, Bucks, UK). The filter was probed with ³²P labelled plasmid pSS1tgXS-FIX (lanes 1 - 7) and subsequently (after stripping the membranes) with plasmid p931 (lanes 1' - 7'). Copy controls of pSS1tgXS-FIX were included on the gel where indicated. Lane 1, control (non-transgenic) sheep DNA; lanes 2 and 3 control DNA plus 1 and 5 copy equivalents of p5'G3'CVI; lanes 4-7, DNA from transgenic sheep 6LL225, 6LL231, 6LL239 and 6LL240. Each transgenic sheep yields Factor IX hybridising bands of 5.95 kb (EcoRI) and 6.05 kb (BamHI), identical in size to those derived from pSS1tgXS-FIX, showing that the 5' ends of the transgenes are intact. Significant hybridisation with sheep Factor IX was not observed. Hybridisation with p931 revealed the predicted 4.4 kb EcoRI and 2.1 kb BamHI fragments. Although the endogenous sheep betalactoglobulin genes contribute to the hybridisation in these bands, the increased intensity in samples from 6LL225, 6LL231 and 6LL240 indicate that these bands derive primarily from the injected betalactoglobulin-Factor IX fusion gene, confirming the integrity of the 3' ends of the construct. HindIII cleaved DNA: lane 1, 12.1 kb purified Sall-XbaI fragment excised from pSS1tgXS-FIX; lanes 2-5 DNA from 6LL225, 6LL231, 6LL239 and 6LL240. The probe was p5'G3'CVI. The hybridising 12.1 kb HindIII fragment (6LL225, 6LL231 and 6LL240), identical in size with the injected fragment indicates a head to tail arrangement; the 15.6 kb fragment common to the same sheep indicates that head to head repeats are also present. These data indicate that in sheep 6LL225, 6LL231 and 6LL240 the Sall-XbaI fragment derived from pSS1tgXS-FIX (the betalactoglobulin-Factor IX fusion gene) has integrated without detectable rearrangement in tandem arrays. In sheep 6LL239 the data is compatible with the integration of a single unrearranged copy of this fragment.

EXAMPLE 6**Transmission of Factor IX Sequences to the Next Generation**

The male transgenic sheep 6LL225 from Example 5 (carrying approximately 40 copies of the Sall-XbaI fragment prepared from pSS1tgXS-FIX) was successfully mated to a number of Finn-Dorset and East Friesland ewes. His progeny were analysed by Southern blotting of DNA prepared from blood samples using the human Factor IX plasmid probe

p5'G3'CVI, as described. A Southern blot of this analysis on some of his progeny is shown in Figure 5a. Lanes numbered 1,3,4,5,6,7,9,11,43,45,48 and 49, DNA from progeny of 6LL225 (designated 7R1, 7R3 etc - 7R49), lane c 211, DNA from control (nontransgenic) sheep 6LL211, lanes 5-211 and 1-211, DNA from sheep 6LL211 plus 5 and 1 copy equivalents of pSS1tgXS-FIX. These data show that transgenic sheep 6LL225 has transmitted the Factor IX sequences to 6 out of 12 of his progeny and, therefore that these sequences have been incorporated into the germline.

EXAMPLE 7

Expression of the Gene Encoding Ovine Beta Lactoglobulin in Transgenic Mice

Transgenic mice were generated essentially by the techniques described in Gordon and Ruddle, in *Methods in Enzymology* Vol 101 (1983), (Eds. Wu, Grossman and Moldave), Academic Press pp411-432. Several transgenic mice carrying the Sall fragment of the clone lambda SS-1 (Fig. 3) were produced. One of these, B-Lac 7, a female was shown to carry 15-20 copies of the Sall fragment. B-lac 7 was mated a number of times and produced a number of offspring which inherited the SS-I sequences.

Milk was obtained from mice 8-12 days after the birth of a litter. This was accomplished by intra-peritoneal injection of 0.3IU oxytocin (Sigma) & 7 mcl/g animal of Hypnorm/Hypnovel (Fleckneil, Vet. Rec. Dec 10 1983, p574), after having previously removed the pups for a four hour period, waiting 20 minutes and then massaging the individual mammary gland by hand. Milk was collected in a 50 mcl capillary tube.

The mouse milk was diluted 1:5 in distilled water, centrifuged briefly in a bench centrifuge to defat and the caseins precipitated by addition of 1N HCl to a final pH of 4.6. After centrifugation in a bench centrifuge the whey proteins were removed, precipitated with 5% trichloroacetic acid and analysed by polyacrylamide gel electrophoresis according to Laemmli (Nature 277, 680-684 (1970). (Figure 6 shows an SDS PAGE Analysis of Murine and Ovine Whey Proteins. Lane 1, marker proteins; 2, normal mouse whey; 3, sheep whey; 4, normal mouse whey; 5, B-lac7 whey; 6, B-lac7 whey (2.5 x 5). The band corresponding to beta lactoglobulin in the marker track and in ovine whey is arrowed.) Anti-sera raised in rabbits against ovine beta-lactoglobulin was used to detect ovine beta-lactoglobulin by Western blotting (Burnett, Anal. Bioches., 112, 195-203, (1981)) on samples resolved by gel electrophoresis. Figure 7 shows a Western Blot Analysis. The Western blot was reacted with rabbit anti-beta-lactoglobulin serum and anti-rabbit Ig peroxidase serum. (Lane 1, marker proteins; 2, sheep whey; 3, B-lac7 whey; 4, normal mouse whey; 5, purified beta-lactoglobulin; 6, Coomassie stained sheep whey (run in parallel)).

This analysis showed that large amounts of beta-lactoglobulin were secreted into mouse milk, indicating that SS-1 was being expressed at high levels in B-lac 7. This clone presumably contains all the necessary sequences to ensure high levels of expression in the mammary gland of a transgenic mouse and can thus be expected to function as efficiently, if not more so, in the homologous species ie in a transgenic sheep. Consequently, fusion genes derived from this clone can also be expected to express (efficiently) in the ovine mammary gland.

EXAMPLE 8

Expression of Human Factor IX in the Milk of Transgenic Ewes

Two female sheep, 6LL231 and 6LL240 (each carrying approximately 10 copies of Sall-XbaI fragment prepared from pSS1tgXS-FIX) were successfully mated to East Friesland rams. After birth the lambs were allowed to suckle naturally for about two weeks to stimulate lactation. Milk (approximately 25 ml from each animal) was collected by hand into sterile plastic containers. Milk from a control (nontransgenic) lactating ewe was also collected. The samples were frozen at -20°C and delivered to the Scottish National Blood Transfusion Service at the Royal Infirmary, Edinburgh, where radioimmunoassays (RIA's) for human Factor IX were performed.

Transgenic and control milk were dialysed against distilled water overnight at 4°C and then freeze-dried.

Freeze-dried sample were resuspended in distilled water and then centrifuged to separate the cream and examined by RIA for the presence of human Factor IX as follows:-

A standard curve, using normal human pooled plasma diluted in RIA buffer (50mM Tris/HCl, 0.25% gelatin, 1% Tween-20, 10mM HCl, pH 7.2) was established using dilutions of 1/10 - 1/1280 (see Figure 8). In order to rule out any interference by milk in the assay, normal pool plasma was similarly diluted in control milk, and a standard curve established. Dilutions of samples of freeze-dried milk from 6LL231 and 6LL240 were assayed. Each tube in the RIA comprised the following:- 50 mcl RIA buffer, 50 mcl sample dilution, 50 mcl rabbit polyclonal anti-Factor IX antibody (Dako-Patts), at a 1/30000 dilution and 50 mcl ¹²⁵I labelled Factor IX. A control for maximum binding was set up comprising 100 mcl RIA buffer, 50 mcl antibody and 50 mcl of 1125 Factor IX. A control for non-specific binding was also set up comprising 150 mcl RIA buffer, 50 mcl 1125 trace.

After overnight incubation 50 mcl of Sepharose-S1000 conjugated with donkey anti-rabbit IgG was mixed in the tubes and the beads recovered by sucrose separation. The RIA assay is sensitive to about 0.125 international units

(iu)/dl. Any sample containing Factor IX above this level will inhibit maximum binding in the RIA.

The % binding in the RIA was plotted against the reciprocal of the dilution factor for the following samples:-

1. Normal pool plasma
2. Normal pool plasma plus milk
3. Freeze dried milk samples from 6LL231 (T1)
4. Freeze dried milk samples from 6LL240 (T2)

These results are shown in Figure 8.

Milk from both 6LL231 (T1) and 6LL240 (T2) exhibited detectable levels of Factor IX at 2.5 iu/dl and 8.0 iu/dl, respectively. No activity was detected in control milk at the level of sensitivity of the assay. These data show that transgenic ewes carrying the betalactoglobulin-factor IX fusion gene (specifically the Sall-XbaI fragment derived from pSS1tgXS-FIX (also generally designated pSS1tgXS-TARG)) express this gene and secrete the human protein into the milk. This establishes a basis for the production of human proteins in this manner.

Claims

1. A transgenic non-human mammal whose genetic material includes a genetic construct comprising:

- (i) a DNA sequence coding for a polypeptide; and
- (ii) a DNA sequence from a milk whey protein gene to direct expression of the DNA sequence coding for the polypeptide in the mammary gland of the mammal when it is an adult female;

wherein a substance comprising the polypeptide is expressed in the milk of the adult female transgenic human mammal.

2. A transgenic mammal as claimed in claim 1, wherein the milk whey protein gene is a β -lactoglobulin gene.

3. A transgenic mammal as claimed in claim 2, wherein the β -lactoglobulin is ovine β -lactoglobulin.

4. A transgenic mammal as claimed in claim 1, 2 or 3, wherein the substance comprising a polypeptide is a protein.

5. A transgenic mammal as claimed in claim 4, wherein the substance comprising a polypeptide is a human blood protein.

6. A transgenic mammal as claimed in claim 4, wherein the substance comprising a polypeptide is a peptide hormone.

7. A transgenic mammal as claimed in claim 5, wherein the substance comprising a polypeptide is a blood coagulation factor or a subunit of a blood coagulation factor.

8. A transgenic mammal as claimed in any one of claims 1 to 5, wherein the substance comprising a polypeptide is an enzyme.

9. A transgenic mammal as claimed in any one of claims 1 to 8, wherein the mammal is a domestic livestock mammal.

10. A transgenic mammal as claimed in claim 9, wherein the mammal is selected from members of the family *Suidae*, members of the genus *Ovis*, members of the genus *Capra*, and members of the genus *Bos*.

11. A transgenic mammal as claimed in claim 10, wherein the mammal is a dairy sheep.

12. A transgenic mammal as claimed in any one of claims 1 to 11, wherein the genetic construct comprises a promoter.

13. A transgenic mammal as claimed in any one of claims 1 to 12, wherein the genetic construct comprises a start site for transcription.

14. A transgenic mammal as claimed in any one of claims 1 to 13, wherein the genetic construct comprises one or more distal 5' regulatory sequences of the milk whey protein gene.

15. A transgenic mammal as claimed in any one of claims 1 to 14, wherein the genetic construct comprises a structural milk whey protein gene sequence optionally including internal regulatory sequences.

16. A transgenic mammal as claimed in any one of claims 1 to 15, wherein the genetic construct comprises a 3' sequence flanking the milk whey protein gene.

17. A transgenic mammal as claimed in any one of claims 1 to 16, wherein the genetic construct comprises a cDNA sequence coding for the polypeptide of interest inserted into the first exon of the gene encoding the milk whey protein.

18. A transgenic mammal as claimed in any one of claims 1 to 17, wherein the genetic construct includes some of the 5' flanking sequences of the gene encoding the milk whey protein.

19. A transgenic mammal as claimed in any one of claims 1 to 18, wherein the genetic construct contains a sequence encoding a signal peptide for the polypeptide of interest.

20. A transgenic mammal as claimed in claim 19, wherein the sequence encoding a signal peptide of the milk whey protein gene is precisely fused to that portion of the DNA sequence that encodes the N-terminal amino acid of the mature polypeptide in the substance produced.

21. A transgenic mammal as claimed in any one of claims 1 to 20, wherein the 3' end of the DNA sequence terminates after its stop codon, but before its own polyA addition site.

22. A method of producing in milk a substance comprising a polypeptide, the method comprising:

(a) producing milk in the mammary gland of an adult, female, non-human mammal whose genetic material includes a genetic construct comprising:

(i) a DNA sequence coding for the polypeptide; and

(ii) a DNA sequence from a milk whey protein gene to direct expression of the DNA sequence coding for the polypeptide; and

(b) collecting the milk produced in step (a).

23. Any novel feature of European Patent Application No. 87904286.9, from which this application is divided.

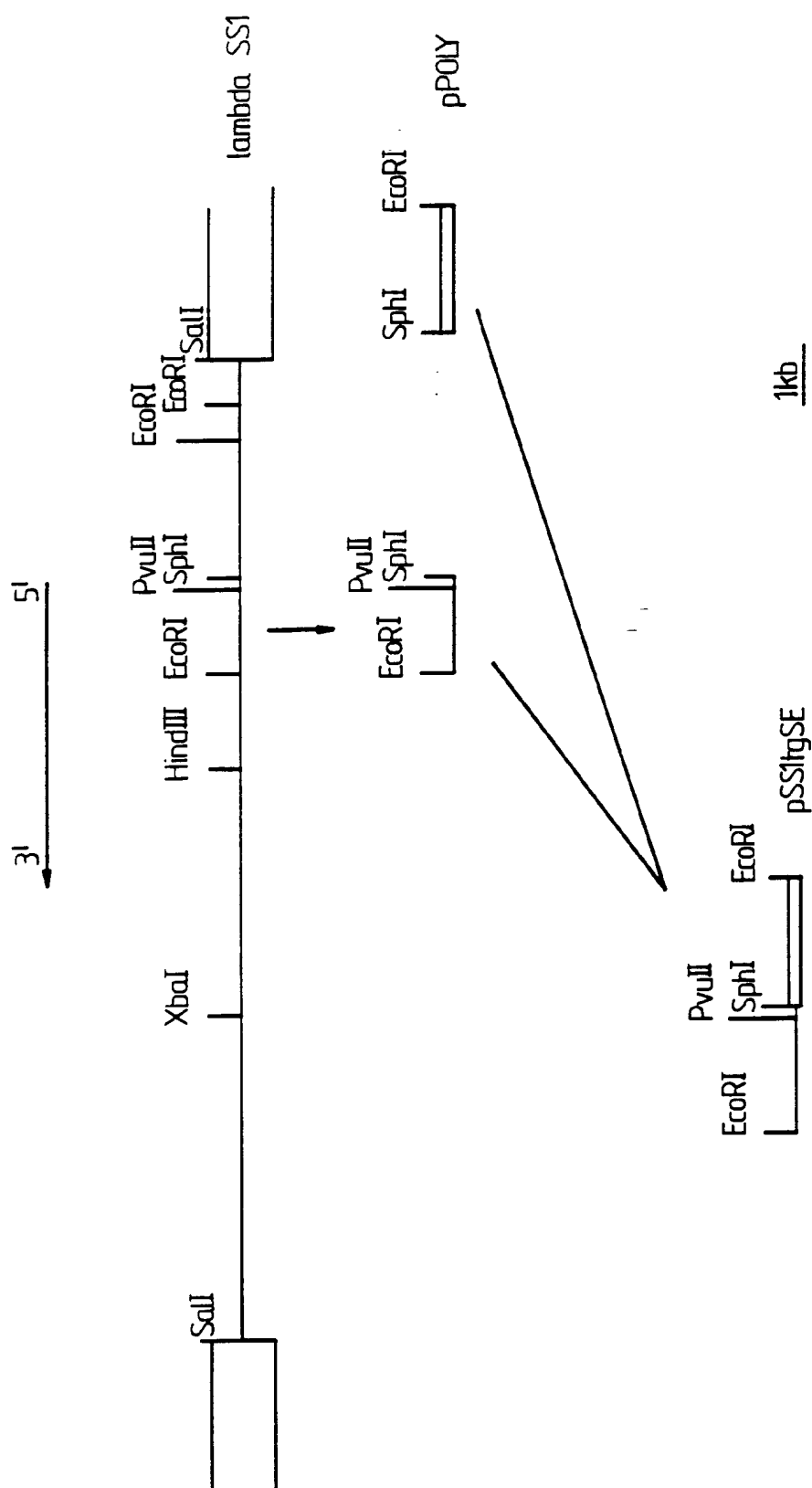


FIG.1.

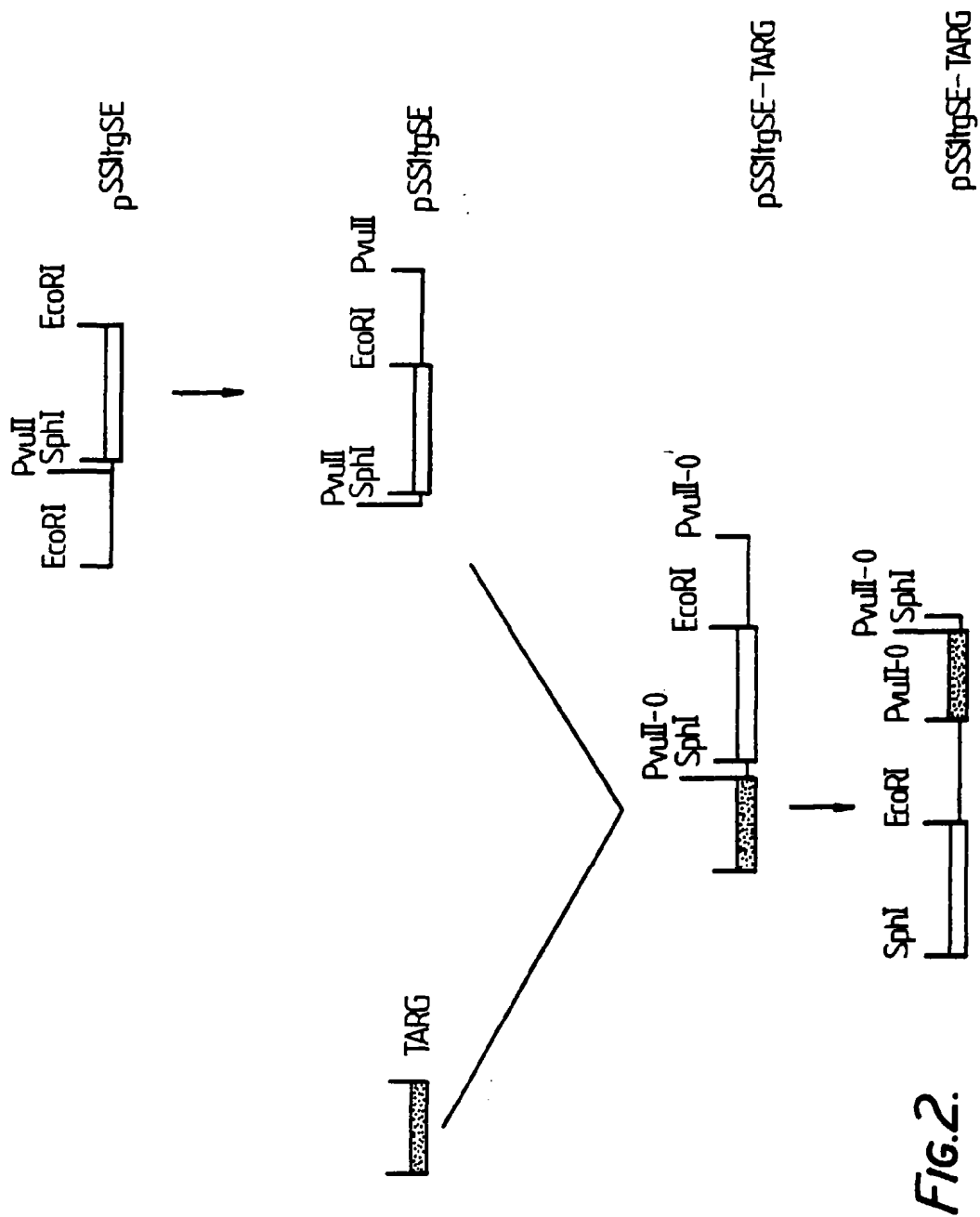


FIG. 2.

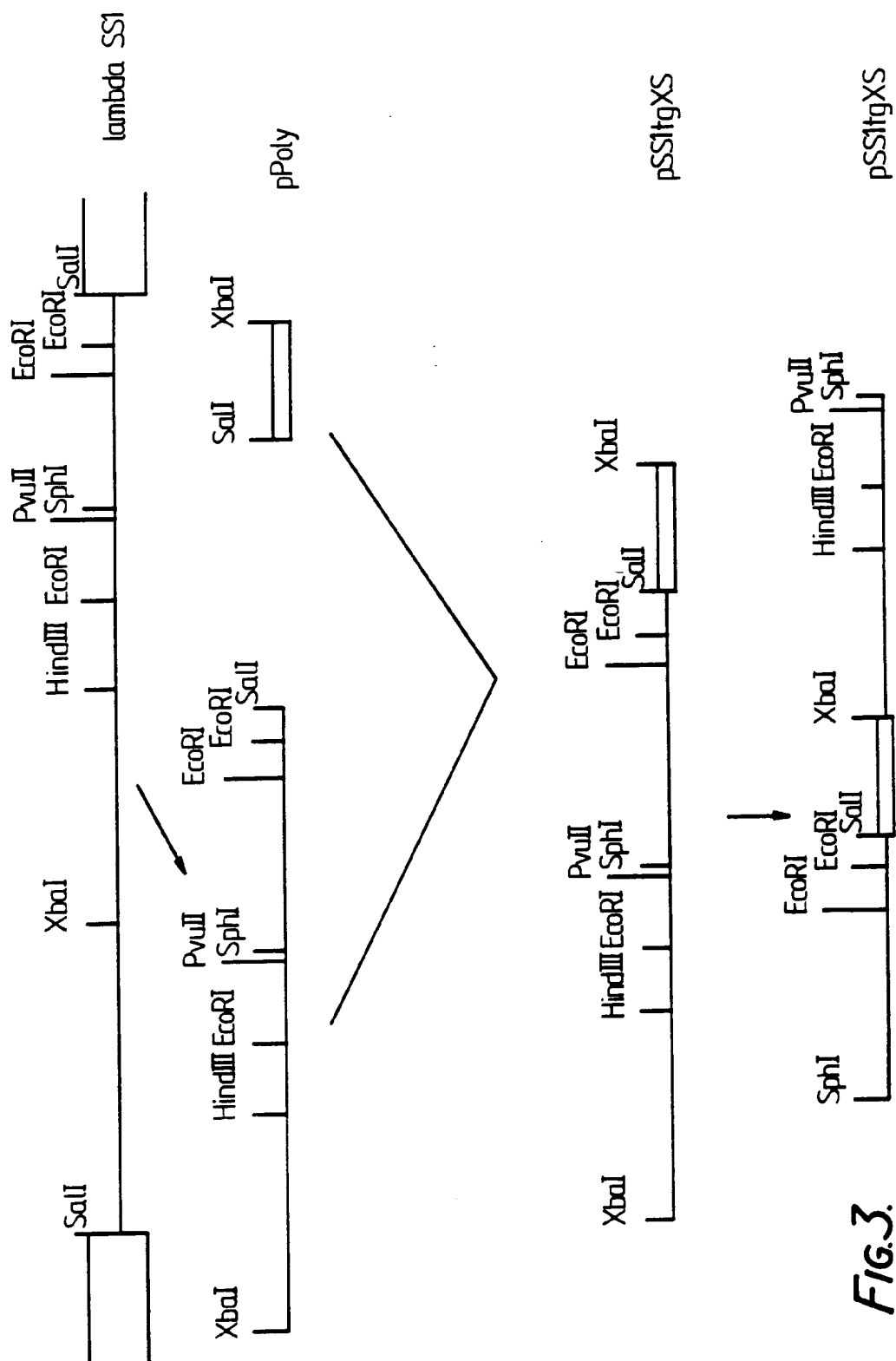


FIG.3.

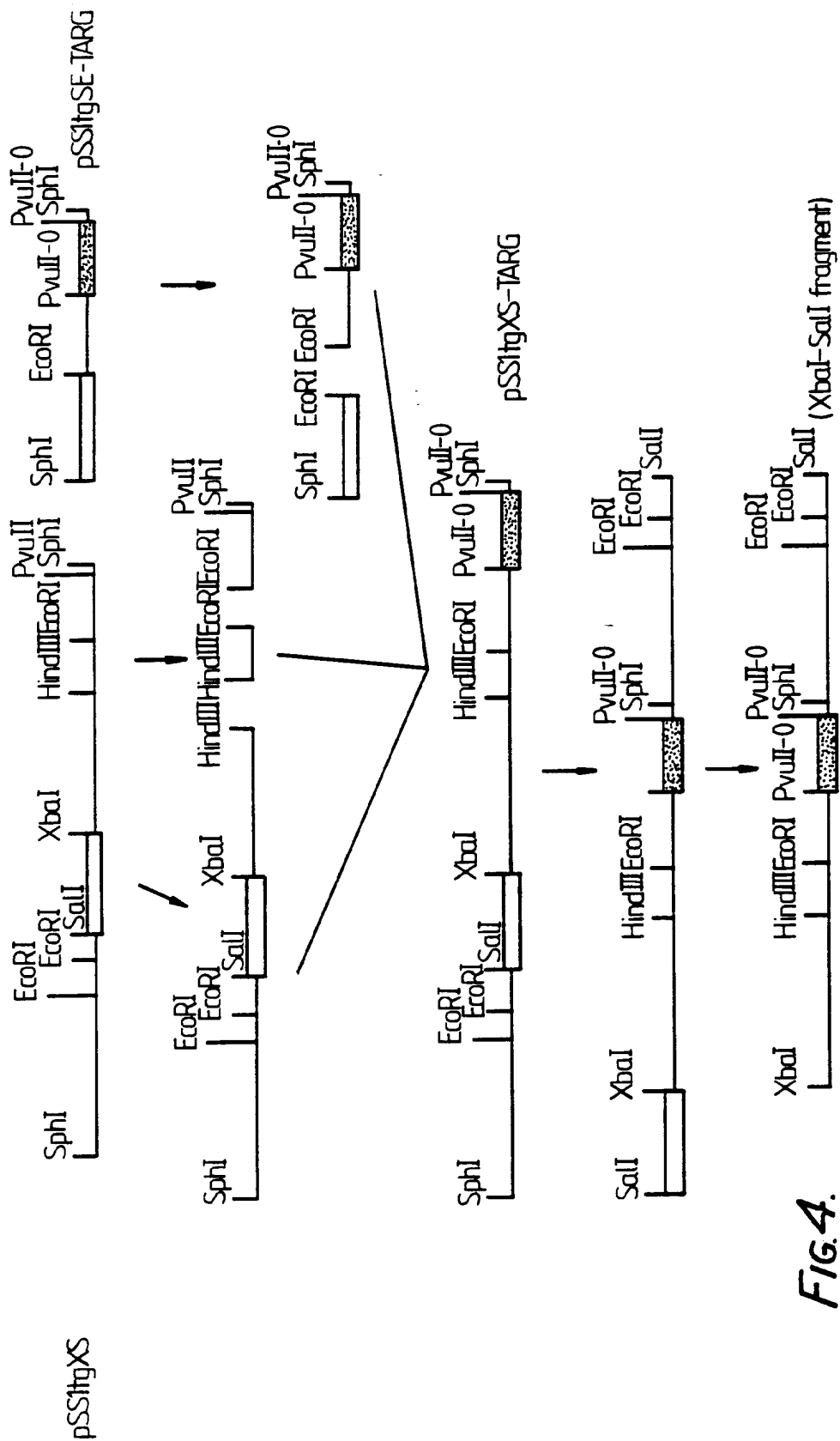


FIG. 4.

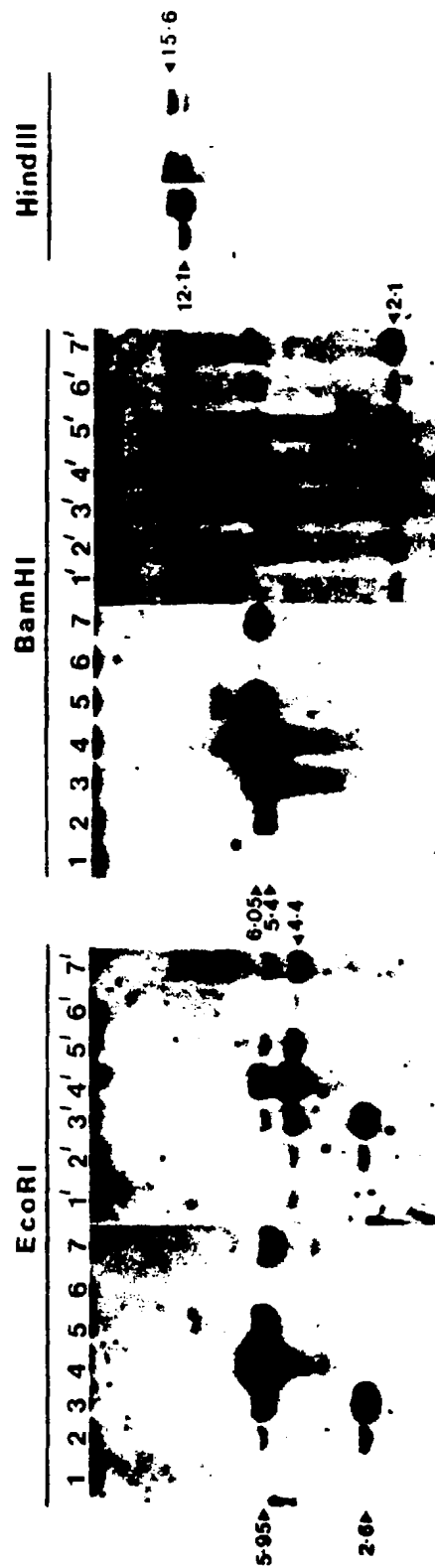


FIG.5.

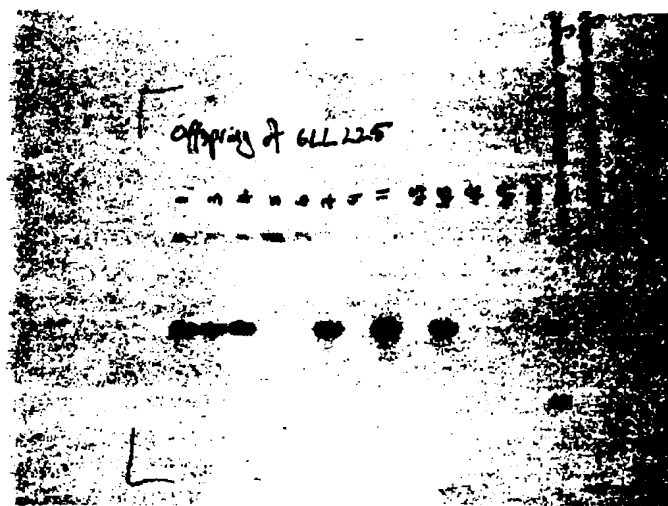


Fig.5a.

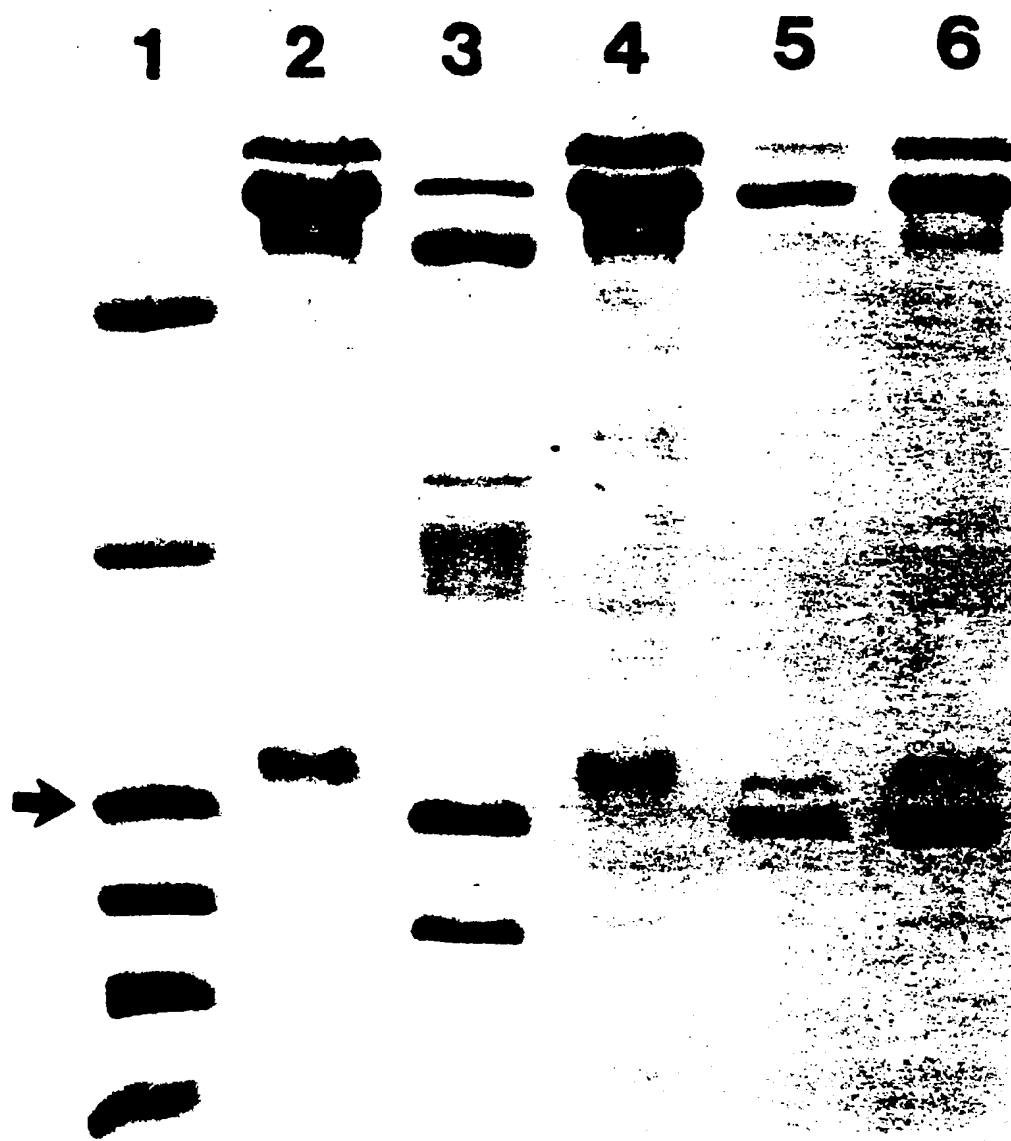


FIG.6.

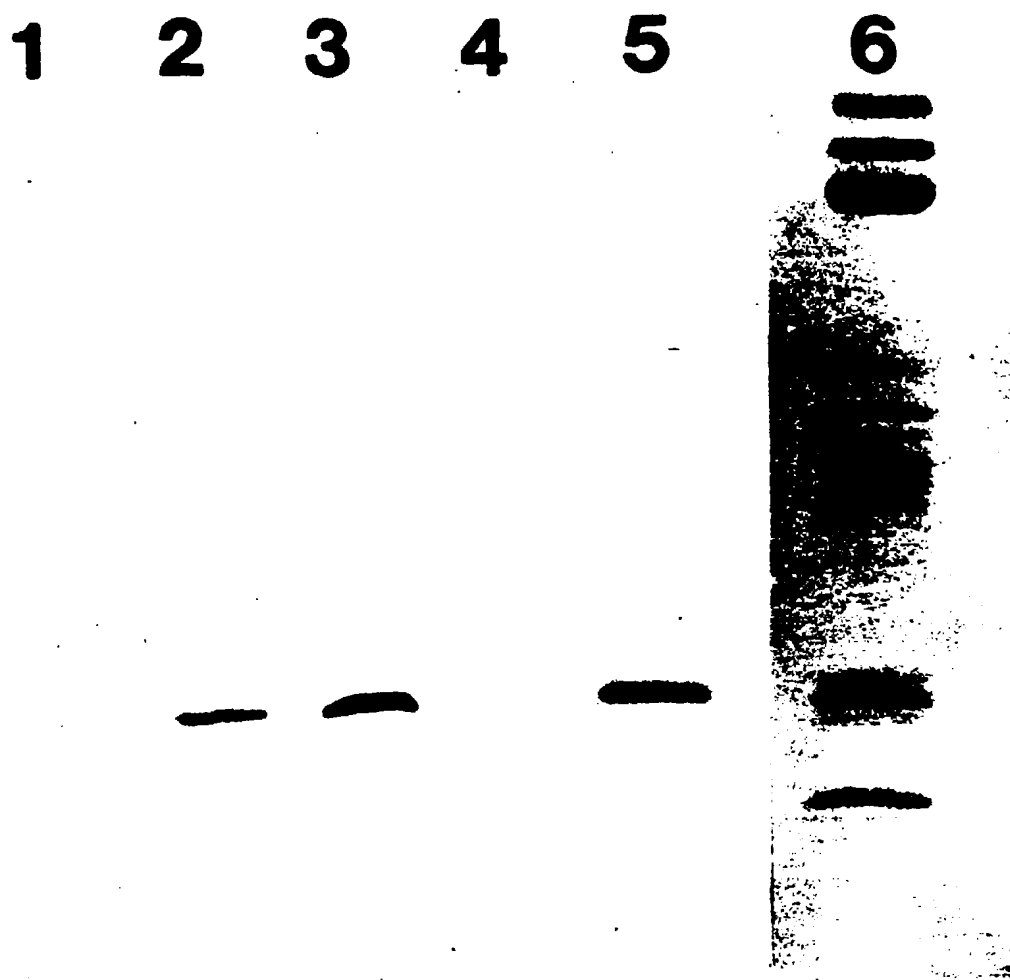


Fig.7

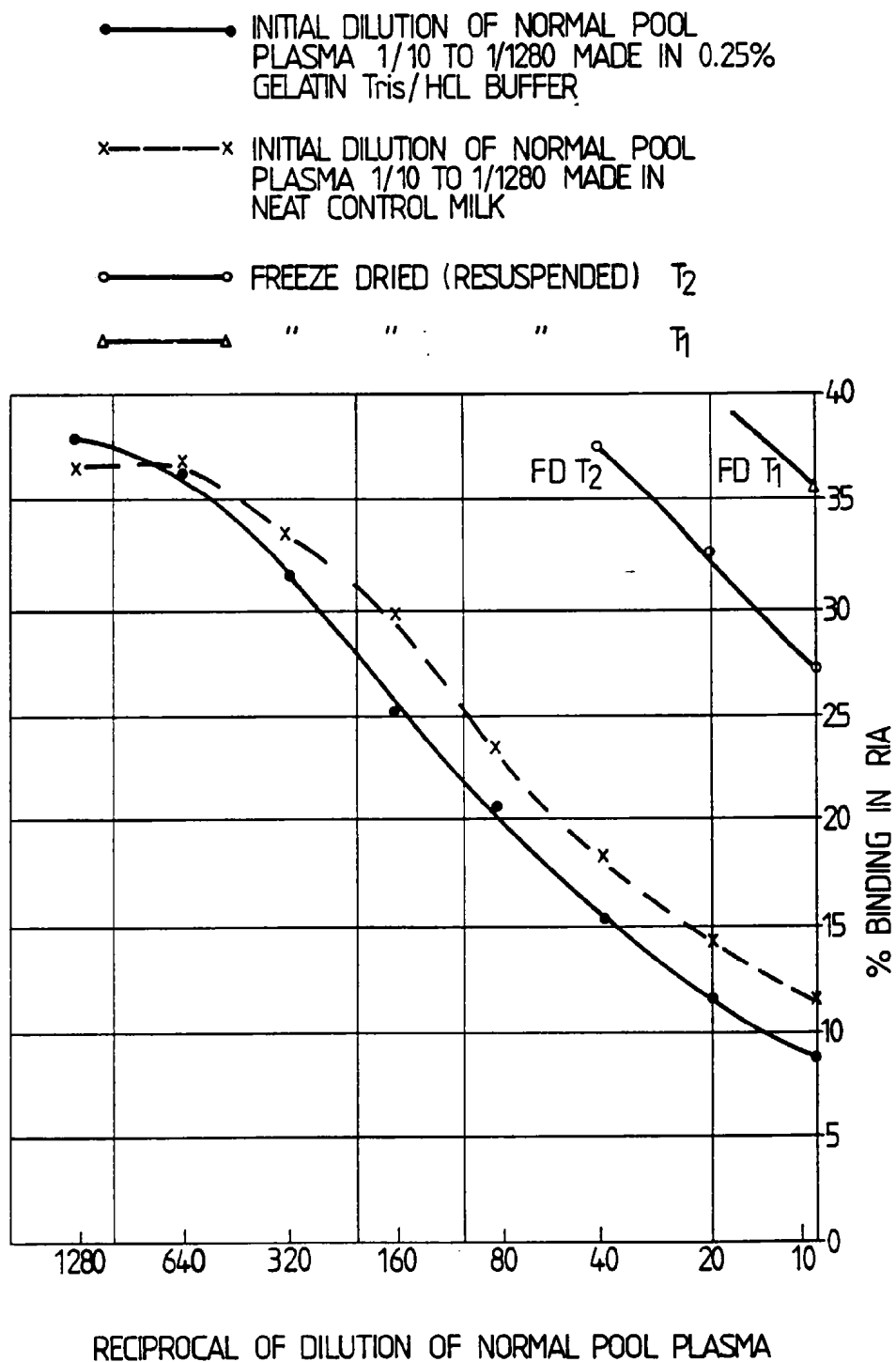


Fig.8.



European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 97 20 0314

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
E	EP 0 264 166 A (INTEGRATED GENETICS, INC.) 20 April 1988 * the whole document * ---	1,3-5, 7-22	C12N15/00 A01K67/027
X	NATURE, vol. 315, 20 June 1985, LONDON GB, pages 628-629, XP002032479 R.H. LOVELL-BADGE: "Transgenic animals: New advances in the field" * page 269, column 2, last paragraph * ---	1,4	
A	NATURE, vol. 315, 20 June 1985, LONDON GB, pages 680--683, XP002032480 R.E. HAMMER ET AL.: "Production of transgenic rabbits, sheep" * the whole document * ---	1-22	
P,X	TRENDS IN BIOTECHNOLOGY, vol. 5, no. 1, January 1987, CAMBRIDGE GB, pages 20-24, XP002032481 A.J. CLARK ET AL.: "Pharmaceuticals from transgenic livestock" * page 22 - page 23 * -----	1,4	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C12N A01K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 6 June 1997	Examiner Cupido, M
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application I : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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